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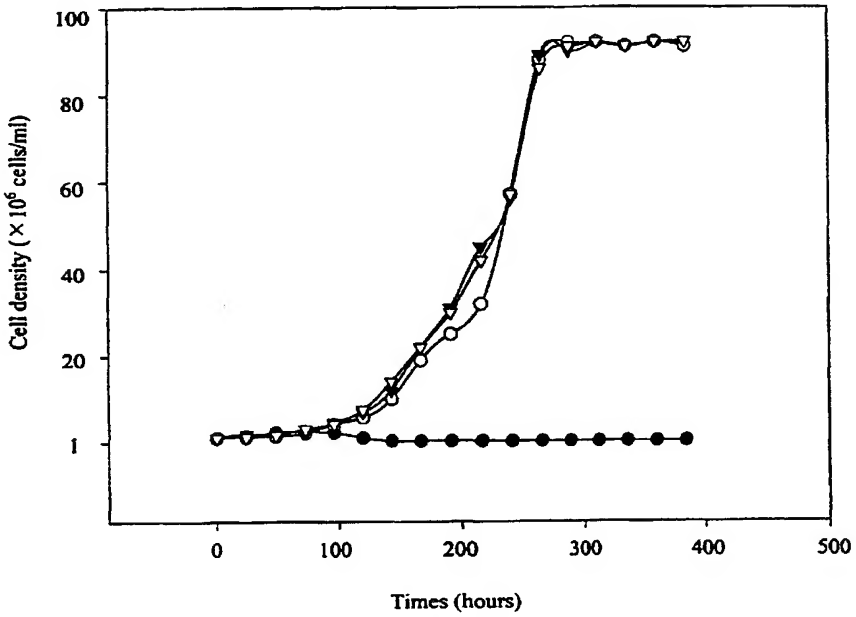
(54) Title: BIOSYNTHESIS OF FOREIGN PROTEINS USING TRANSFORMED MICROALGAE



(57) Abstract: The present invention relates to an economical method for biosynthesis of intended foreign proteins using transformed microalgae, that is to say, it relates to a method of using transformed microalgae as a bioreactor where economical biosynthesis of foreign protein is possible by transforming protoplast of microalgae such as *Chlorella ellipsoidea* with DNA vector containing intended foreign protein gene and then culturing it in a large scale. In particular, Sh ble gene, which is resistant to phleomycin, is used as a selection marker in the present invention.

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BIOSYNTHESIS OF FOREIGN PROTEINS USING TRANSFORMED MICROALGAE

FIELD OF THE INVENTION

1. FILED OF THE INVENTION

The present invention relates to a method for biosynthesis of foreign protein using transformed microalgae. More particularly, it relates to a method for biosynthesis of foreign proteins by transforming microalgae protoplasts with DNA vector containing intended foreign protein gene, and then culturing it in a large scale.

2. DESCRIPTION OF THE PRIOR ARTS

Escherichia coli is the most widely used heterologous expression system, but the bacterium has some limitations including; i) poor or no expression of certain proteins, ii) some recombinant proteins lack biological activity, iii) some recombinant proteins are toxic to *Escherichia coli*, and iv) some recombinant proteins form insoluble inclusion bodies. Similar problems can occur with yeast expression systems. Cultured mammalian and insect cells have been used to solve these problems, but these systems can be expensive because of the cost of media, equipment and requirement for extensive purification procedures.

Therefore, the present inventors made an investigation on chlorella transformation as a new heterologous overexpression system, which could substitute for *Escherichia coli,* to solve the above-described problems.

As a result thereof, we have found that microalgae expression system was

more economical than cell culture or animal or plant expression system because the microalgae had simpler metabolic pathway than those of animals or plants had and could be cultured in a large scale using an aquarium with light and carbon dioxide. Moreover, the fact that microalgae has post-translational modification process unlike *Escherichia coli* indicates that the biological activity of foreign protein expressed in microalgae should be more similar to that of naturally occurring protein. Under this circumstance, we intended to develop microalgae overexpression system for producing foreign proteins.

There had been attempts to transform the *Chlorella* species, one of microalgae. Jarvis and Brown described the transient expression of luciferase in protoplasts of *Chlorella ellipsoidea* (Jarvis, E. E., and Brown, L. M. 1991. Transient expression of firefly luciferase in protoplasts of the green alga *Chlorella ellipsoidea*, Current Genetics 19, 317-321) and Dawson *et al.* found that nitrate reductase-deficient mutants of *Chlorella sorokiniana* could be rescued by transforming them with nitrate reductase gene isolated from *Chlorella vulgaris*(Dawson, H. N., Burlingame, R., and Cannons, A. C. 1997. Stable transformation of *Chlorella*: Rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. Current Microbiology 35, 356-362). However, these experiments described only transient expression or expression of protein genes originated from chlorella species.

Therefore, the present inventors made investigations on a method for biosynthesizing foreign protein by transforming protoplasts of microalgae with a vector DNA containing the genes originated from organisms other than microalgae and then culturing them in a large scale. As a result thereof, we found out that objects described above could be reached with this method.

SUMMARY OF THE INVENTION

The object of present invention is to provide a method for the stable expression of a foreign protein in a microalgae overexpression system.

To achieve the above object, the method of the present invention is characterized in that which comprises the steps of; (i) obtaining protoplast of microalgae; (ii) preparing a vector containing genes coding desired proteins, said genes originated from organisms other than microalgae; (iii) introducing the vector into the protoplast to give transformed protoplast and (iv) culturing the transformed microalgae to produce the desired protein.

Also, the method of the present invention could further comprise another step of selecting transformed cells with antibiotics between step (iii) and step (iv) other than the above steps.

The above and other objects, features and application of the present invention will be apparent to those of ordinary skill by the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows photographs of *Chlorella ellipsoidea* cells stained with calcofluor white before (a) and after (b) enzyme treatment for cell wall removal observed by fluorescent microscope.

Figure 2 shows a photograph of expression of GFP in transformed *Chlorella ellipsoidea* observed by fluorescent microscope.

Figure 3 shows a schematic diagram of the transformation vector pCTV.

Figure 4 shows the growth of transformed and non-transformed *Chlorella*

ellipsoidea cultured in medium containing or not containing phleomycin.

Figure 5 shows the results of PCR amplification and Southern blot analysis of flounder growth hormone(hereinafter fGH) gene and Sh ble gene inserted into genomic DNA of transformed *Chlorella ellipsoidea.*

In figure 5, panel A shows the result of PCR amplification and Southern blot analysis for fGH and panel B shows the result of PCR amplification and Southern blot analysis of Sh ble; lane 1 shows molecular weight size marker and lane 2 shows transformed *Chlorella ellipsoidea* and lane 3 shows non-transformed *Chlorella ellipsoidea* and lane 4 shows fGH and Sh ble gene fragments digested from pBluescript SK+.

Figure 6 shows the result of Western blot analysis of fGH expressed in tranformed *Chlorella ellipsoidea.* In figure 6, 1ane 1 shows molecular weight size marker and lane 2 shows the Glutathione-S-transferase(hereinafter, GST)-fGH fusion protein used for production of antibody and lane 3 shows the total protein isolated from non-transformed *Chlorella ellipsoidea* and lane 4 shows the total protein isolated from transformed *Chlorella ellipsoidea.*

Figure 7 shows the result of Western blot analysis showing the amount of fGH expressed in transformed *Chlorella ellipsoidea.* In figure 7, lane M shows molecular weight size marker and lane 1 & 2 show the 10μg of GST-fGH fusion protein and lane 3 & 4 show the fGH isolated from 10ml transformed *Chlorella ellipsoidea* and lane 5 & 6 show the 10μg of GST protein.

Figure 8 shows the results of Western blot analysis showing fGH accumulated on *Brachionus plicatilis* and *Artemia naupilus*, both of which were fed with *Chlorella ellipsoidea* transformed with fGH. In figure 8, lane 1~4 show

Brachionus plicatilis 30, 60, 90 and 120 minutes, respectively after feeding with transformed *Chlorella ellipsoidea* and lane 6~8 show *Artemia naupilus* 30, 60 and 90 minutes, respectively after feeding with transformed *Chlorella ellipsoidea*.

Figure 9 shows the growth promotion of flounder by *Chlorella ellipsoidea* transformed with fGH. In figure 9, open bars indicate the growth promotion by transformed *Chlorella ellipsoidea,* and filled bars indicate the growth promotion by non-transformed *Chlorella ellipsoidea,* and vertical line indicates standard deviation, and lower cases indicate significant differences ($p<0.05$).

Figure 10 shows the growth promotion of flounder fries after 30 days feeding of *Brachionus plicatilis* and *Artemia naupilus* that had been fed for 1 hour with *Chlorella ellipsoidea* transformed with fGH.

DETAILED EXPLANATION OF THE INVENTION

Throughout the specification and claims, the term of foreign protein is used to mean any protein originated from organisms different from host microalgae, and it includes its active fragments, variants, and analogues as long as they retain its original biological activity. The term of "foreign gene" is used to indicate any nucleic acid sequence, regardless of its source(natural or synthetic), coding the foreign protein as defined above, and may include, DNA, RNA, cDNA or their variants resulting form base deletion, substitution or insertion, as long as they still code for the foreign protein having its biological activity.

Chlorella ellipsoidea is an attractive organism for the production of complex proteins because of its eukaryotic characteristics and low cost for large-scale culture. The inventors report the first functional expression of a foreign protein, the flounder

growth hormone(fGH) in *Chlorella ellipsoidea*, and the growth promotion of fish by feeding them this transformed chlorella. Protoplasts of *Chlorella ellipsoidea* were transformed with a vector containing the fGH gene under the control of the cauliflower mosaic virus 35S promoter and the phleomycin resistance *Sh ble* gene under the control of the *Chlamydomonas RBCS2* gene promoter. PCR amplification and Southern blot analysis of the *fGH* and *Sh ble* genes from chromosomal DNA isolated from the transformants confirmed stable integration of introduced DNA. Western blot analysis indicated that the fGH protein was expressed in the transformed chlorella. The introduced DNA and the expressed fGH were detected after seven successive transfers in media devoid of phleomycin. The transformed chlorella cells were first fed to zooplanktons to remove the cellulose cell wall, and then the planktons were fed to flounder fries. These fish showed a 25% increase in total length and width after 30 days of feeding when compared to control fish. These results indicate that *Chlorella ellipsoidea* can be used to produce valuable proteins at low cost.

In the present invention, the green fluorescence from chlorella transformed with the *GFP* gene and the phleomycin resistance of chlorella transformed with *Sh ble* gene indicate the functional expression of these proteins. The biological activity of the recombinant fGH was confirmed by feeding flounder fry. Thus, in the present invention, it was confirmed that the microalgae transformed with flounder growth hormone gene could express the hormone in a biologically active form. Therefore, it's possible to produce valuable proteins for medicine and industry from transformed microalgae. In particular, microalgae could be produced with simple equipment and low cost, and a method of isolation and purification of expressed

proteins therefrom is also simple, so that the cost of producing protein could be significantly reduced.

Further, this invention describes the successful use of the *Sh ble* gene as a selectable marker for *Chlorella ellipsoidea* transformation, the first demonstration of stable gene integration and expression of a biologically active foreign protein in the transformed *Chlorella ellipsoidea*. The results indicate that *Chlorella ellipsoidea* can be used to produce proteins of scientific or pharmacological use.

The microalgae used in the present invention are not particularly limited but the technique can be applied to other algae including *Chlorella* from sea and fresh water such as *Chlorella ellipsoidea*, *Chlorella sorokiniana* and *Chlorella vulgaris, Chlamydomonas, Volvox, Cheatoceros, Phaeodactylum, Skeletonema, Navicula, Caloneise, Nitzschia, Thalassiosira, Amphora, Nannochloris, Nannochloropsis, Tetraselmis, Dunaliella, Spirulina, Microcystis, Oscillatoria, Tricodesminus, Isochryosis, Pavlova, Dinophyceae* and the like.

The foreign protein gene used in the present invention is the flounder growth hormone gene. However, other genes originated from bacteria, fungi, virus, animals, plants or fishes could be used for overexpression by using the present invention.

And, vector production, cloning, transformation of host by vector, selection and culture of transformant, and the recovering process of the desired protein after culture are known to those of skilled in the art.

The following examples are provided to illustrate the present invention, which should not be construed to limit the scope of the present invention.

[EXAMPLE 1] Culture and protoplast formation of *Chlorella ellipsoidea*

Chlorella epillsoidea was obtained from the Korea Marine Microalgae Culture Center of Pukyong National University (Strain No. KMCC C-20). Cells were inoculated in fresh f/2 medium(Guillard, R. R. L., and Ryther, J. H. 1962. Studies on marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea(Cleve) Gran. Can. J. Microbiol. 3, 229-239) containing 50 μg/ml each of chloramphenicol and streptomycin at an initial concentration of 1×10^6 cells/ml and cultured at 25°C, 18:6 hour photoperiod under a 3000 lux fluorescent lamp. Cells were harvested for protoplast formation 8-9 days after inoculation when the cell count reached 1-2 $\times10^8$ cells/ml. Cells (50ml) were centrifuged for 5minutes at 1,500×g, washed once with 25mM phosphate buffer (pH 6.0), and suspended in 5ml of phosphate buffer containing 0.6M sorbitol, 0.6M mannitol, 4% (w/v) cellulase(Calbiochem, USA), 2%(w/v) macerase(Calbiochem), and 50 units pectinase(Sigma Chemicals, USA). The cell suspension was incubated at 25°C for 16 hours in the dark with gentle shaking.

Protoplast formation of *Chlorella ellipsoidea* was confirmed in two ways. In an osmo-stability test, the number of enzyme treated cells in distilled water decreased from 1.7 $\times10^6$ cells/ml to 1.0 $\times10^5$ cells/ml in 8 hours, whereas no change occurred in the number of untreated chlorella. This result was confirmed by calcofluor white staining(Maeda, H., and Ishida, N. 1967. Specificity of binding of hexapyranosyl polysaccharides with fluorescent brightner. J. Biochem. 62, 276-278). Over 80% of enzyme-treated cells were red in contrast to untreated cells that were blue when visualized by fluorescent microscope (see Fig. 1); these findings indicated complete removal of the cellulose component of the cell wall, to which

calcofluor white binds.

[EXAMPLE 2] Preparation of pMinGFP and the Expression of GFP

As a first step to develop a chlorella transformation system, a small 5 kb binary vector was constructed from the plant transformation vector Bin19(Bevan, M. 1984. Binary Agrobacterium vectors for plant transformation. Nucleic Acids Res. 12, 8711-8712). The new vector called pMIN, contains the oriV origin for replication in both *E. coli* and *Agrobacterium*, the *npt II* gene for kanamycin resistance, the *trfA* gene for DNA replication, and the right and left border T-DNA elements for integration. The subsequent cloning of a DNA fragment containing the cauliflower mosaic virus 35S promoter to direct expression of the green fluorescent protein (GFP) produced a vector pMinGFP for use in higher plant and algae transformation. Chlorella protoplasts were transformed with pMinGFP by polyethylene treatment and the expression of GFP was measured. After 7 days of culture in f/2 medium without selection for transformation, a small number of chlorella cells exhibited GFP fluorescence, whereas non-transformed chlorella cells did not (see Fig. 2).

[EXAMPLE 3] Cloning the *fGH* gene

A flounder cDNA library was constructed with the Lambda ZAP-II cDNA synthesis kit (Stratagene, USA) using total mRNA isolated from the Japanese flounder pituitary gland. The titer of the amplified library was 3×10^9 pfu/ml and a 1μl aliquot was used for PCR amplification. The DNA fragment amplified with fGH-AN (5'-CGGGATCCCAGCCAATCACAGA-3') and fGH-AC (5'-CGGGCTACAGAATTC-3') primers was cloned into the pGEM-T vector (Promega, USA) for sequence confirmation. A *BamHI*/*Nde*I fragment was subcloned into the pGEX-3X vector (Amersham Pharmacia Biotech, USA) for glutathion-S-

transferase-fGH (GST-fGH) fusion protein expression; this fusion protein was used for polyclonal antibody production.

[EXAMPLE 4] Preparation of pMinfGH

Growth hormone genes have been cloned from several fish species and their growth-enhancing effects have been observed in transgenic fish. The growth hormone gene (*fGH*) from the Japanese flounder, *Paralichthys olivaceus*, the major aquaculture fish in Korea, was used to transform chlorella. The *fGH* gene was cloned by PCR amplification of a flounder pituitary cDNA library, using the fGH-N primer (5'-CGGGATCCGGTCAGTCCCTTATGCAGCCAATCACA-3') and fGH-C primer (5'-AAAAGCTCGAGCTCTTGGCGGAG-3') (Watahiki, M., Yamamoto, M., Yamakawa, M., Tanaka, M. & Nakashima, K. 1989. Conserved and uniques amino acid residues in the domains of the growth hormone: flounder growth hormone deduced from the cDNA sequence has the minimal size in the growth hormone prolactin gene family. J. Biol. Chem. 264, 312-316). Replacement of the GFP gene in pMinGFP vector by the 560 bp PCR product resulted in vector pMinfGH.

[EXAMPLE 5] Preperation of pCTV

We used the *Sh ble* gene, originated from *Streptoalloteichus hindustamus*, which encodes a small protein (13.7kDa) that confers resistance to tallysomycin, bleomycin, phleomycin, and zeomycin by binding to the antibiotics and inhibiting their DNA cleaving activities. To determine if *Chlorella ellipsoidea* was inhibited by phleomycin, the alga was cultured in f/2 medium containing different concentrations of phleomycin; Reduced growth occurred in media containing 0.1 or 0.5 μg/ml phleomycin, and the alga failed to grow in media containing more than 1

μg/ml phleomycin. Thus, the *Sh ble* gene that confers resistance to phleomycin is suitable to select transformed chlorella. The *Sh ble* coding region and upstream *Chlamydomonas reinhardtii RBCS2* promoter were amplified from the plasmid pSP109(Lumbreras, V., Stevens, D. R., & Purton, S. 1998. Efficient foreign gene expression in Chlamydomonas reinhardtii mediated by an endogenous intron. Plant J. 14, 441-447) with ble-N primer (5'-AAACTCGAGGGCGCGCCAGAAGGAGC-3') and ble-C primer (5'-AAACTCGAGAATTCGAGGTCGGTACC-3'). The 880 bp PCR product was digested with *Xho I* and subcloned into pMinfGH to construct the chlorella transformation vector pCTV (see Fig. 3).

[EXAMPLE 6] Transformation of *Chlorella ellipsoidea* with pCTV vector

Chlorella protoplasts(1×10^8) were centrifuged at 400×g for 5 minutes, resuspended in 5 ml of f/2 medium containing 0.6M sorbitol/mannitol, centrifuged at 400×g for 5 minutes, and resuspended in 1ml of 0.6 M sorbitol/mannitol solution containing 0.05M $CaCl_2$. Then, 1×10^8 protoplasts in 0.4ml were placed in a fresh microcentrifuge tube and 5μg of pCTV vector was added with 25μg calf thymus DNA(Sigma Chemicals). After 15 minutes incubation at room temperature, 200μl of PNC[0.8 M NaCl, 0.05 M $CaCl_2$, 40 % PEG 4000(Sigma Chemicals)] was added and mixed gently for 30 minutes at room temperature. Then, 0.6 ml f/2 medium supplemented with 0.6 M sorbitol/mannitol, 1% yeast extract and 1% glucose was added, and the cells were incubated at 25°C for 12 hours in the dark for cell wall regeneration. The cells were transferred to fresh f/2 medium containing phleomycin (1μg/ml) and cultured as described above.

Detectable growth occurred by 5 days and the cell growth reached stationary phase by 15 days. In contrast, no detectable growth occurred in non-transformed

protoplasts(see Fig. 4). The slow growth of the transformed chlorella cells was consistent with the preliminary transformation experiments with pMinGFP, where only a small percentage (2%) of the cells displayed green fluorescence. When the transformed chlorella cells in the stationary phase were transferred to fresh f/2 medium or f/2 medium containing phleomycin, no detectable growth differences occurred. Furthermore, the growth rates in the two media were similar to the growth of non-transformed chlorella in f/2 medium lacking phleomycin (see Fig. 4). These results indicate that the introduced DNA has no effect on chlorella growth; also the transformed cells did not exhibit any morphological changes.

[EXAMPLE 7] Stable integration of introduced DNA

Stable integration of introduced DNA into chromosomal DNA is a prerequisite for use of chlorella as an expression system. PCR and Southern analyses were performed to determine if the introduced DNA was integrated into the chlorella chromosomal DNA.

(A). DNA isolation.

Approximately $3x10^8$ transformed cells were pelleted from 3 ml of culture, resuspended in 500 μl of CTAB buffer[250ml: hexadecyltrimethylammonium bromide(CTAB) 5g, 1M Tris(pH 8.0) 25ml, NaCl 20.45g, EDTA 1.68g, β-mercaptoethanol(2%)] and incubated at 65°C for 1 hour, and then extracted with an equal volume of phenol/chloroform. The aqueous phase recovered after 5 minutes centrifugation at 3,000xg was extracted several times and chromosomal DNA was precipitated with ethanol, pelleted and resuspended in 30 μl of TE buffer.

(B). PCR and Southern blot analysis.

fGH-N/fGH-C and ble-N/ble-C primer pairs were used to amplify the *fGH* gene and

the *Sh ble* gene from isolated chromosomal DNA, respectively. 200ng of chromosomal DNA and 100pmole of each of the primers were added to 50 μl reactants, and subjected to 30 cycles of 1 minute denaturation at 94°C, 30 seconds annealing at 54 or 57°C for the fGH and the *Sh ble* genes, respectively, 1 minute extension at 72°C followed by 5 minutes extension at 72°C. Probes for Southern blot were synthesized using the DIG-DNA labeling kit (Boehringer Mannheim, Germany).

PCR products of the expected size were produced only with DNA isolated from transformed chlorella. These DNA fragments were identified by Southern analyses with probes specific to the *fGH* or *Sh ble* genes(see Fig. 5). The stability of the integrated DNA was confirmed by PCR amplification of the two genes from the chromosomal DNA isolated from chlorella after seven serial transfers into medium lacking phleomycin.

[EXAMPLE 8] Expression of fGH in tranformed *Chlorella ellipsoidea*

fGH expression was tested by Western analysis as described hereinafter. Transformed *Chlorella ellipsoidea* was harvested from 3 ml of culture containing 10^8 to 10^9 cells by centrifuging for 5 minutes at 17,000 x g. The cells were homogenized in liquid nitrogen, resuspended in 20 μl of sample loading buffer[1mM EDTA, 250mM Tris-Cl (pH 6.8), 4 % SDS, 2 % β- mercaptoethanol, 0.2 % bromophenyl blue, 50 % glycerol], and boiled for 10 minutes. The sample was centrifuged for 10 minutes at 12,000 x g and the supernatant was electrophoresed on a 15 % SDS-PAGE. Also, protein extracts prepared from non-transformed chlorella were separated by SDS-PAGE. Western blot analysis was conducted by standard procedures. Protein extracts separated from tramsformed and non-transformed

chlorella by SDS-PAGE were transferred onto nitrocellulose membranes. The final dilution of polyclonal antibody against fGH was 1:3,000 and alkaline phosphatase-conjugated anti-mouse IgG was used as the secondary antibody.

The 20kDa fGH was present in transformed chlorella but absent in non-transformed cells (see Fig. 6).

One requirement for a successful expression system is that the foreign protein be produced at high level. The amount of fGH expressed in transformed chlorella was determined by an Enzyme Linked Immunosorbent Assay (ELISA) and Western blots with purified GST, GST-fGH fusion protein and extract from transformed chlorella using polyclonal antibody against GST-fGH fusion protein(see Fig. 7). About 400ng of fGH was obtained from 1×10^8 stationary phase cells (400μg of total protein in 1ml culture). The yield is equivalent to 400μg fGH per litter of cultured chlorella assuming a final cell count of $1x10^8$ cells /ml. Considering the low cost of culture medium for the alga, this system could be used to produce eukaryotic proteins, especially proteins of pharmaceutical importance.

[EXAMPLE 8] Biological activity test

Although chlorella can not be directly fed to fish and crustacean larvae because of the high cellulose content in their cell walls, chlorella have been used to mass culture zooplanktons, which contain cellulase. Also it is known that fish can take up proteins in feed by pinocytosis and there are reports of fish growth promotion by the oral administration of recombinant mammalian and fish growth hormone.

Thus, four day old flounder larvae were grouped into 1000 fish each in a 300 litter tank filled with 200 litter of sea water. Rotifers (*Brachionus plicatilis*) and brine shrimp (*Artemia nauplius*) were used to accumulate the growth hormone and to

remove the cellulose from chlorella cell wall. Zooplanktons were starved for one day after hatching and provided with 3x108 cells/ml of transformed and non-transformed chlorella for one hour. Western analysis confirmed that the fGH in the alga accumulated in zooplankton bodies by 1 hour of feeding; after 1 hour fGH was degraded and disappeared 2 hours after feeding (see Fig. 8). The flounder larvae were fed once a day with the rotifers for 10 days and then with a mixture of the rotifers and the brine shrimp for 5 days, followed by 15 days feeding with the brine shrimp. The final counts of the rotifer and the brine shrimp were 10 and 5 individuals/ml, respectively. Four day old flounder fries were cultured for 30 days with zooplanktons enriched for 1 hour with transformed and non-transformed chlorella. The lengths of the fish larvae were measured after 10 day feeding and both the length and width of the larvae were measured after 30 day feeding. Fifty randomly selected fish were measured from each of three replicates containing 1000 fish. As shown in Fig. 9, the length of the fish differed significantly after 10 days and had a 25% increase in both length and after 30 days (see Fig 10).

Although preferred embodiments of the present invention have been described in detail herein above, it should be clearly understood that many variations and/or modifications of the basic inventive concepts herein taught which may appear to those skilled in the art will still fall with in the spirit and scope of the present invention identified in the appended claims.

CLAIMS

1. A method for biosynthesizing of a foreign desired protein in microalgae which comprises the steps of

(i) obtaining protoplast of microalgae;

(ii) preparing a vector containing a gene coding for the foreign desired protein, said gene being originated from organisms other than microalgae;

(iii) introducing the vector into the protoplast to give transformed protoplast; and

(iv) culturing the transformed microalgae to produce the desired protein.

2. The method according to claim 1, further comprising the step, following the culturing step, of recovering the protein from the microalgae.

3. The method according to claim 1, wherein said method further comprises a step of selecting transformed cells with antibiotics between step(iii) and step(iv).

4. The method according to claim 1, wherein said method further comprises a step of egenerating cell walls of the transformed protoplast between step(iii) and step(iv).

5. The method according to claim 3, wherein said vector contains Sh ble gene as a selection marker for transformant and wherein the antibiotics are selected from the group consisting of phleomycin, tallysomycin, bleomycin, and zeomycin .

6. The method according to claim 1, wherein said vector contains promoter which is

selected from the group consisting of cauliflower mosaic virus 35S promoter and the *Chlamydomonas RBCS2* gene promoter.

7. The method according to claim 1, wherein said microalgae is one of *Chlorella* from sea and fresh water, *Chlamydomonas, Volvox, Cheatoceros, Phaeodactylum, Skeletonema, Navicula, Caloneise, Nitzschia, Thalassiosira, Amphora, Nannochloris, Nannochloropsis, Tetraselmis, Dunaliella, Spirulina, Microcystis, Oscillatoria, Tricodesminus, Isochryosis, Pavlova or Dinophyceae.*

8. The method according to claim 1, wherein said desired foreign protein is originated from bacteria, fungi, virus, animals, plants or fishes.

9. The method according to claim 8, wherein said desired foreign protein is flounder growth hormone.

10. A recombinant DNA vector for biosynthesizing a desired foreign proteine in microalgae, comprising a gene coding for the desired foreign protein and Sh ble gene as a selection marker for transformant.

11. A trasformed microalgae for biosynthesizing a desired foreign protein, wherein the genome thereof is integrated with the desired foreign gene and Sh ble gene.

12. The trasformed microalgae according to claim 11, wherein said microalgae can express the desired foreign protein and Sh ble protein.

13. A desired foreign protein produced by expression of the foreign desired gene in the trasformed microalgae according to claim 11 or 12.

14. A method for breeding the aminal with the microalgae according to claim 11 or the protein according to claim 13.

FIG. 1

(a) (b)

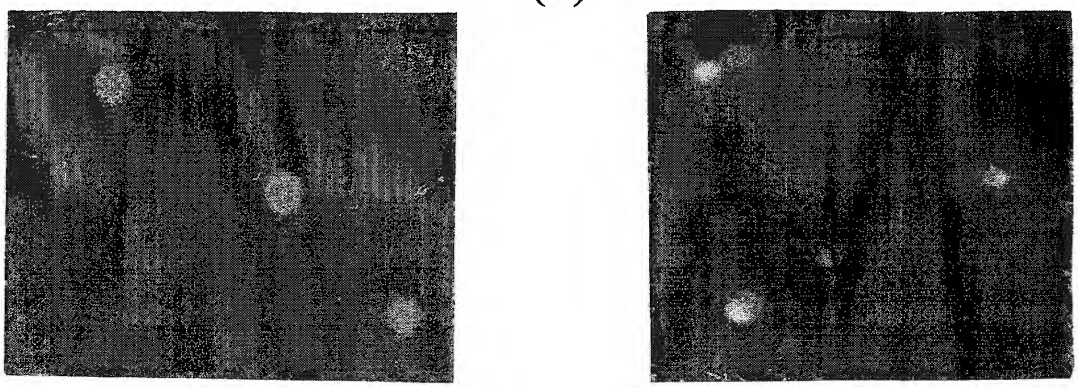


FIG. 2

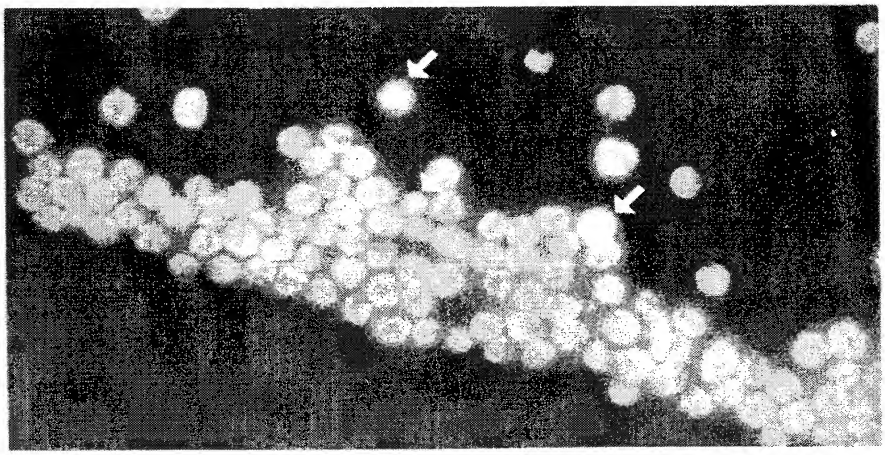


FIG. 3

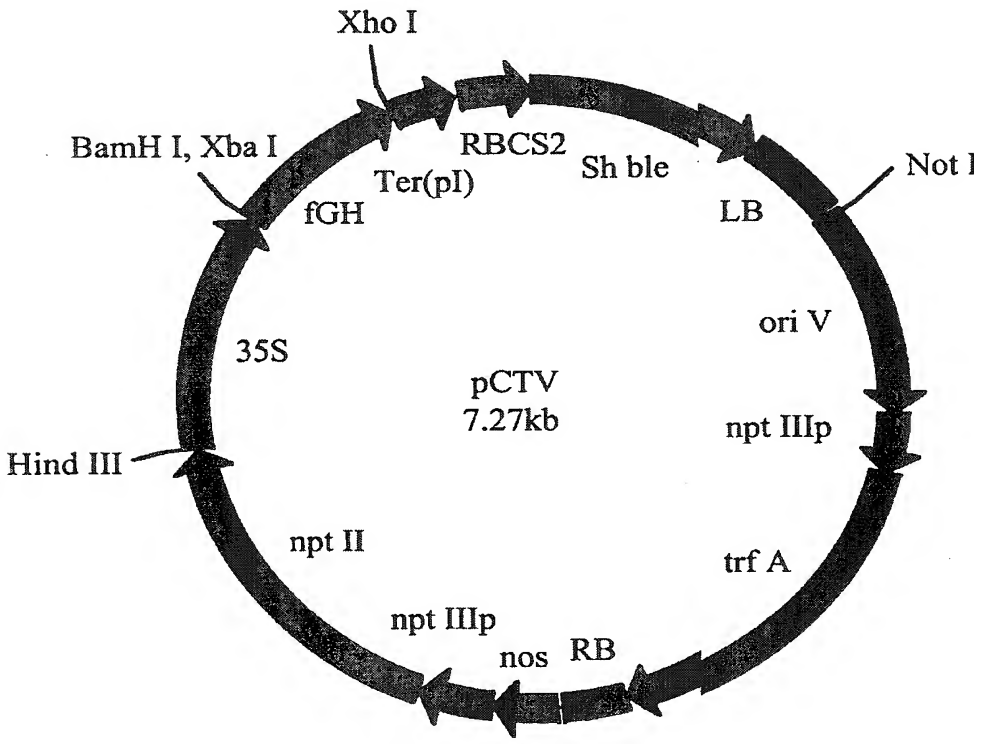

Xho I
BamH I, Xba I
RBCS2
Sh ble
Not I
Ter(pI)
fGH
LB
ori V
35S
pCTV
7.27kb
npt IIIp
Hind III
npt II
trf A
npt IIIp
nos
RB

FIG. 4

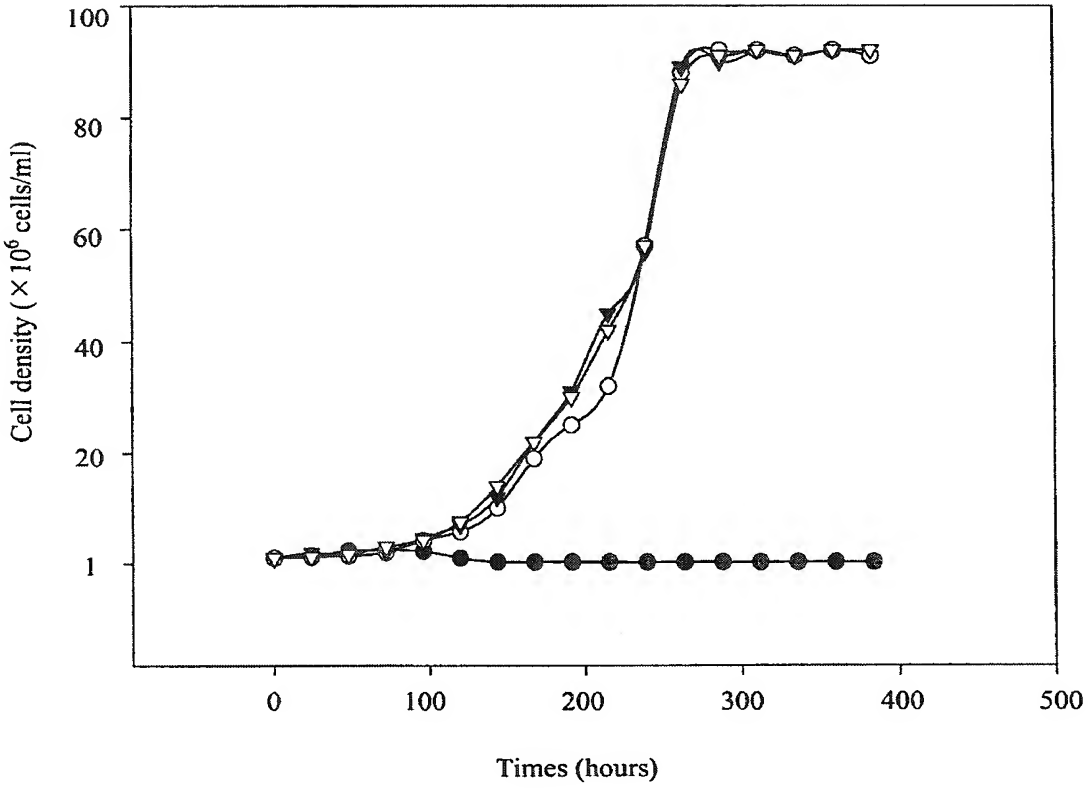
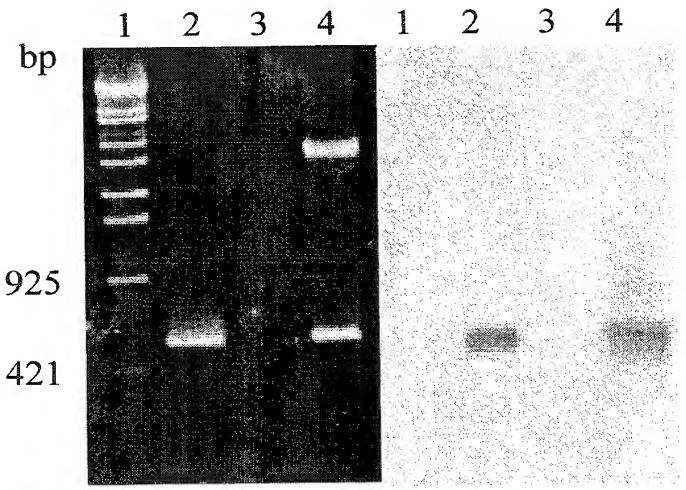

100
80
60
40
20
1
Cell density ($\times 10^6$ cells/ml)
0
100
200
300
400
500
Times (hours)

FIG. 5

(A)



(B)

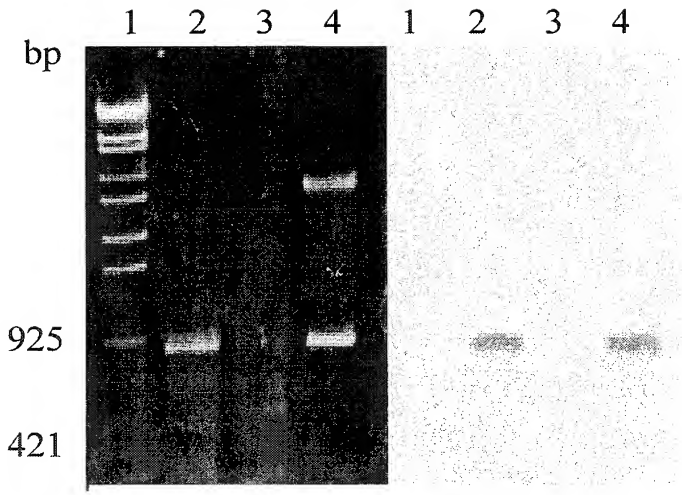


FIG. 6

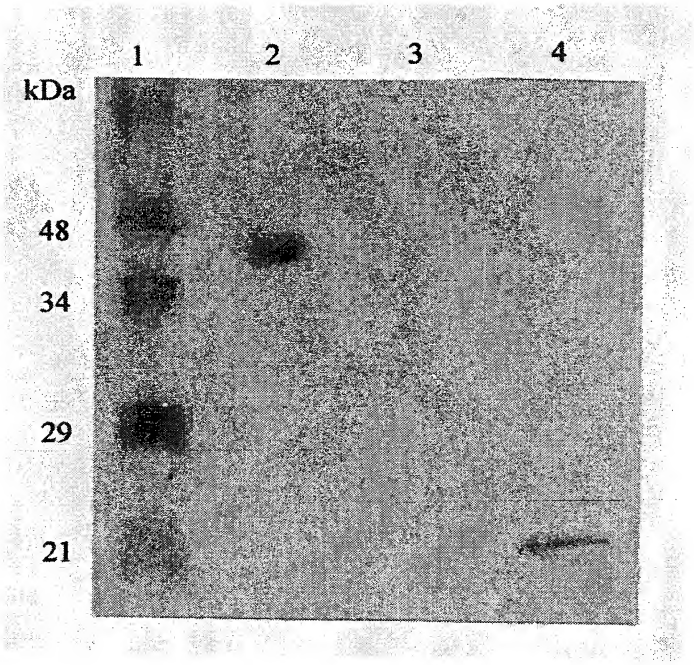


FIG. 7

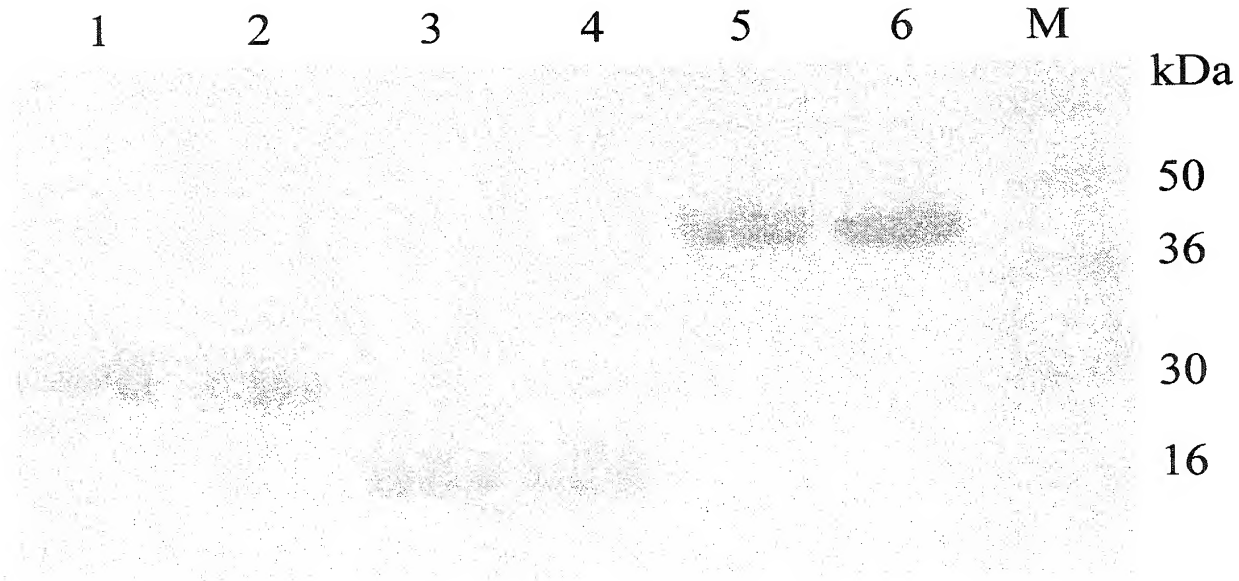


FIG. 8

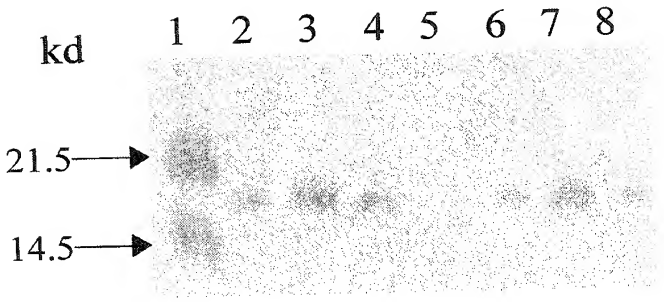

kd
1 2 3 4 5 6 7 8
21.5
14.5

FIG. 9

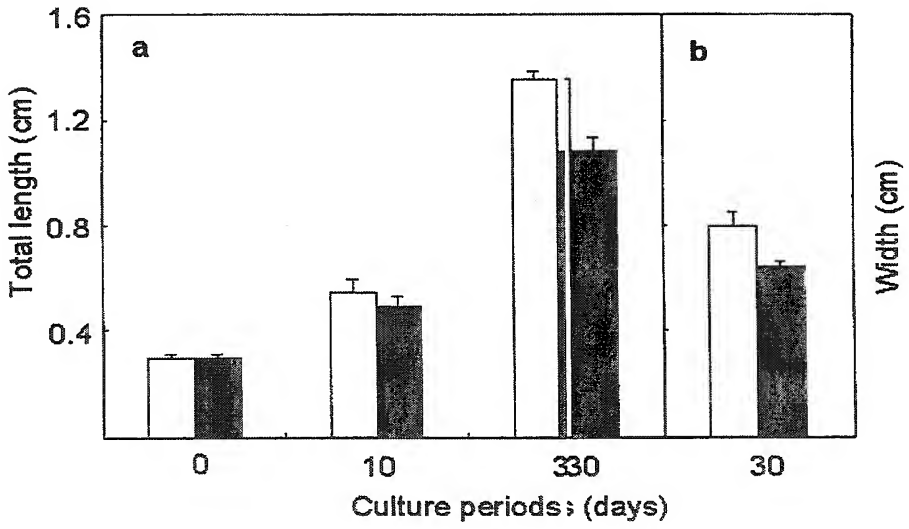
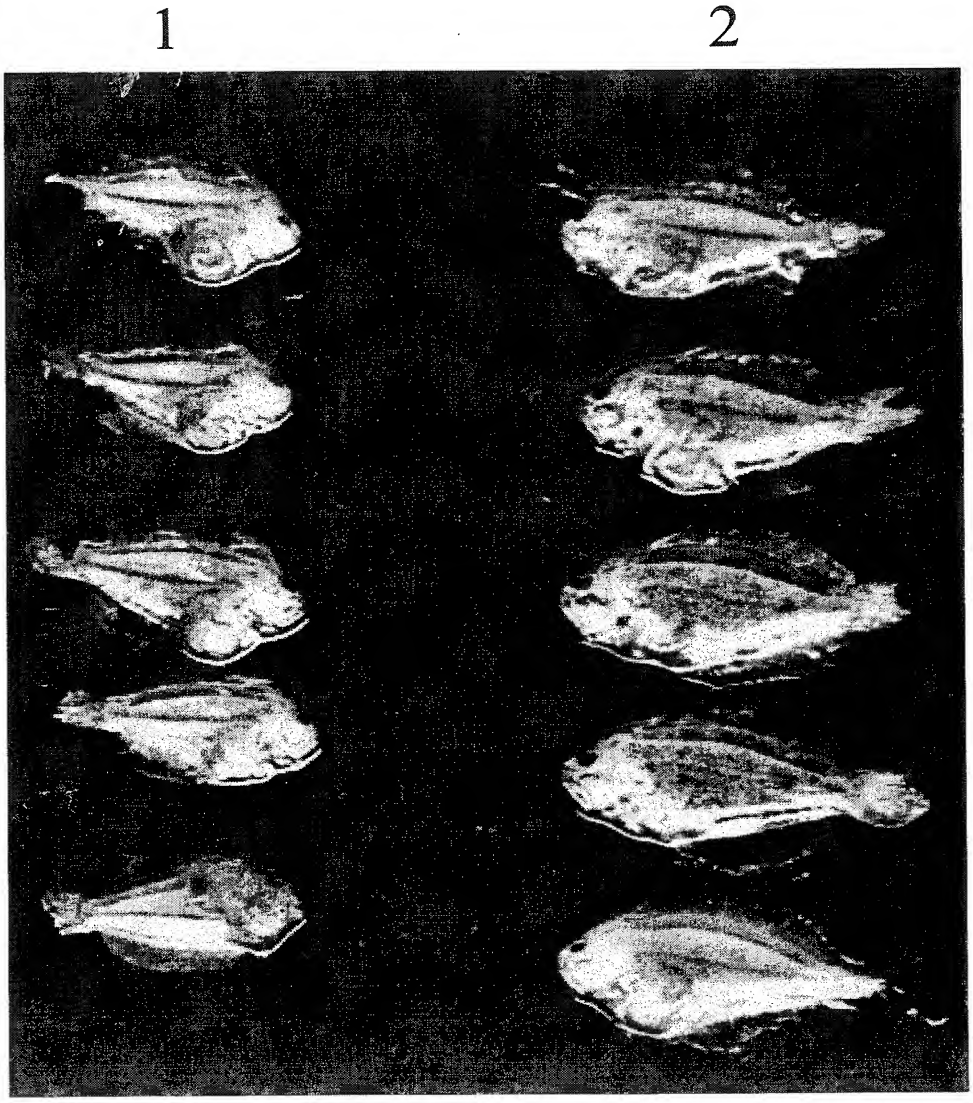

1.6
1.2
0.8
0.4
Total length (cm)
a
b
Width (cm)
0
10
330
30
Culture periods (days)

FIG. 10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 00/00233

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C 12 N 15/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C 12 N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0915101 A1 (HIH. BIOCENTER INC.) 12 May 1999 (12.05.99) totality. ----	1,2,8,9

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2000 (30.06.2000)	31 July 2000 (31.07.2000)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Wolf Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 00/00233

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP A1 915101	12-05-1999	AU A1 64212/98	20-10-1998
		WO A1 9842748	01-10-1998